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<p>(21) International Application Number: PCT/US95/07186 (22) International Filing Date: 7 June 1995 (07.06.95) (30) Priority Data: 258,672 13 June 1994 (13.06.94) US (60) Parent Application or Grant (63) Related by Continuation US 258,672 (CIP) Filed on 13 June 1994 (13.06.94) (71)(72) Applicant and Inventor: ROYER, Garfield, P. [US/US]; 260 Orrtanna Road, Cashtown, PA 17310 (US). (74) Agent: BYRNE, Thomas, E.; Nixon & Vanderhye P.C., 8th floor, 1100 North Glebe Road, Arlington, VA 22201-4714 (US).</p>		<p>(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published With international search report.</p>
<p>(54) Title: A BIO-ERODIBLE MATRIX FOR THE CONTROLLED RELEASE OF MEDICINALS AND THE ASSAY OF HYDROLYTIC ENZYMES</p> <p>(57) Abstract</p> <p>A bio-erodible matrix (2) for the controlled release of medicinals including protein therapeutics is disclosed. A method for controlled drug release is also disclosed.</p> <div data-bbox="824 1199 1360 1738"><p>The diagram shows a circular cross-section of a bio-erodible matrix, labeled with the number 2. Inside the matrix, there are several wavy lines representing the matrix structure. There are also several small, oval-shaped components, some of which are labeled with the number 4. Some of these components are further labeled with the number 6. There are also some components labeled with the number 8. Some of these components are further labeled with the number 10. Some of these components are further labeled with the number 12. The diagram illustrates the internal structure of the matrix and the distribution of the components.</p></div>		

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**A BIO-ERODIBLE MATRIX FOR THE
CONTROLLED RELEASE OF MEDICINALS AND
THE ASSAY OF HYDROLYTIC ENZYMES**

5

FIELD OF THE INVENTION

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This invention relates generally to a bio-erodible delivery system which enables timed release of medicinals including proteins and small molecules.

BACKGROUND OF THE INVENTION

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The rapid progress in recombinant DNA technology has provided researchers and clinicians with a variety of newly discovered proteins in amounts sufficient to enable laboratory and clinical study (Cytokines, A. Meager, Prentis Hall, 1991). Proteins either currently being administered by physicians or under
20 investigation include growth factors, interferons, colony stimulating factors, and interleukins. In nature these molecules may act locally as paracrine agents; i.e., they interact with and activate nearby cells. Further, they can be pleiotropic, i.e., they can activate or stimulate more than one kind of cell.

25 Delivery of these highly potent molecules for treatment of disease remains a challenge. Serious toxicity, low maximum tolerated doses (MTD), and limited therapeutic windows have been observed. As noted above, since some of these molecules are paracrine agents, localized delivery is another issue (Golumbek, P.T., et al, Cancer Research, 53, 5341 (1993)). As an example, systemic use of
30 certain colony stimulating factors may result in autoimmunity and tissue damage from intense inflammatory reactions. Temporary relief of illness may be followed by permanent damage to the immune system.

Many novel proteins now being investigated for clinical use have very short half-lives. Clearance from the circulation can occur in a few hours or even a few minutes. Hence, prolonged release of effective doses below the MTD would be advantageous.

5

Recombinant hormones such as BGH are widely used in dairy cattle. BGH is currently administered biweekly by injection. Controlled release of protein components in veterinary vaccines is desirable. Reduction of the frequency of injection and improvement in performance of the bio-active protein would be advantageous.

Bio-erodible polymers have been used for encapsulation of numerous classes of drugs (U.S. Patent No. 4,349,530; Royer, G.P., et al, J. Parenteral Science & Technol., 37, 34 (1983); Lee, T.K., et al, Science, 213, 233 (1981);
15 WO91/06287 (1991); WO93/25221 (1993), all of which are hereby incorporated by reference). Synthetic polymers and copolymers of lactic acid and glycolic acid have been extensively investigated (U.S. Patent 5,122,367; Langer, Science, 249, 1927 (1990); U.S. Patent 4,983,393 (1991)). Autologous albumin and gelatin are also exemplified in the literature (U.S. Patent No. 4,349,530; Royer,
20 G.P., et al, J. Parenteral Science & Technol., 37, 34 (1983); Lee, T.K., et al, Science, 213, 233 (1981)). Cross-linking with glutaraldehyde is known to stabilize albumin and gelatin matrices. Glutaraldehyde, however, is non-specific in its reaction with proteins. As a result, the protein drug can be inactivated or covalently bound to the matrix components. The latter reaction lowers the
25 effective amount of deliverable drug or creates an antigenic molecule. A negative consequence of the latter chemical reaction is the development of autoimmunity.

U.S. Patent 4,349,530 discloses implants, microbeads and microcapsules comprising cross-linked but physically-native albumin and a biologically active substance. The implants, microbeads and microcapsules may contain an inactive form of a protease capable of dissolving albumin.

5

U.S. Patent 4,983,393 discloses a composition for use as an intra-vaginal insert comprising agarose, agar, saline solution glycosaminoglycans, collagen, fibrin and an enzyme.

10 Failures of conventional delivery systems for proteins are typically attributable to lack of design for controlled release, denaturation of the protein in the matrix, adverse immunological reactions, or chemical modification of the medicinal during formulation.

15

OBJECTS OF THE INVENTION

It is an object of the invention to provide a bio-erodible delivery system which enables timed release of medicinals.

20 It is an object of the invention to provide a delivery system for proteins which does not alter the biological activity of the proteins.

It is a further object of the invention to provide a delivery system where the release profile is easily altered.

25

It is a further object of the invention to provide a means for assaying naturally-occurring hydrolases, such as proteases, in vitro.

SUMMARY OF THE INVENTION

The subject invention relates to a medicinal delivery system comprising
(i) at least one protein selected from the group consisting of gelatin and albumin,
5 (ii) a polymeric stabilizer and/or an external cross-linker, and optionally (iii) an
enzyme capable of degrading said protein or said polymeric stabilizer, wherein
said system is stabilized by said protein being cross-linked with said polymeric
stabilizer and/or said external cross-linker.

The invention also includes a sustained release delivery system
10 comprising

(a) a first gel matrix comprising (i) a protein selected from the group
consisting of gelatin and albumin, and (i) a polymeric stabilizer and/or an
external cross-linker, and (iii) a medicinal protein wherein said first gel matrix is
stabilized by said protein being cross-linked to said polymeric stabilizer and/or
15 said external cross-linker, and

(b) a second gel matrix comprising (i) a protein selected from the group
consisting of gelatin and albumin, and (ii) a polymeric stabilizer and/or an
external cross-linker, and (iii) an enzyme capable of degrading said protein or
said polymeric stabilizer, wherein said second gel matrix is stabilized by said
20 protein being cross-linked to said polymeric stabilizer and/or said external cross-
linker.

Also provided by the invention is a medicinal delivery system comprising
(i) at least one matrix protein selected from the group consisting of gelatin and
albumin, and (ii) a polymeric stabilizer and/or an external cross-linker, wherein
said system is stabilized by said protein being cross-linked with said polymeric
stabilizer and/or said external cross-linker, and (iii) an enzyme capable of
degrading said protein or said polymeric stabilizer embedded on the surface of
said system.

Delivery systems of the invention for sustained release comprise at least two gel matrices wherein at least two of said gel matrices have different levels of cross-linking (internal or external), gel density and/or enzyme.

5 The invention also includes a method for obtaining sustained release of a medicinal comprising administering the delivery systems of the invention to a mammal.

10 Additionally, the invention includes a method of synthesizing a drug delivery system comprising the steps of mixing matrix protein, protected medicinal protein, hydrolytic enzyme, and polymeric stabilizer and shaping the mixture into a gel matrix, and optionally curing said gel matrix with an external cross-linker.

15 The invention also provides a method for assaying hydrolytic enzymes in vitro by the release of compounds, such as polymeric chromophores (fluorophores) in the presence of hydrolases.

BRIEF DESCRIPTION OF THE DRAWINGS

20

FIGURE 1 is a schematic representation of a gel matrix for the timed release of proteins (gelatin molecules not shown);

FIGURE 2 shows periodate oxidation of polysaccharides;

25

FIGURE 3 shows companion beads containing hydrolytic enzyme;

FIGURE 4 shows an externally catalyzed erosion of protein beads;

FIGURE 5 shows an implant or capsule for oral delivery;

FIGURE 6 presents schemes for the modification of amino groups in therapeutic proteins and enzymes; and

5

FIGURE 7 is an idealized profile of programmed release: Class I - contains high levels of enzyme and low matrix density and cross-linking; Class II - moderate enzyme concentration, density, and degree of cross-linking; Class III - low enzyme concentration and in a highly cross-linked, dense matrix.

10

FIGURE 8 shows an enzymograph. Digestion of substrate layers of varying thickness during a preset time period in strips or multi-well plates. After the elapsed time, strips/plates are washed and the position devoid of substrate color or fluorescence indicates the amount of enzyme present in the sample.

15

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a biopolymer gel matrix 2 designed for the controlled release of medicinals 6 including proteins and small molecules.

20 The hydrophilic, non-immunogenic gel matrix consists of (i) one or more proteins, such as gelatin (collagen) and/or albumin 4, (ii) a polymeric stabilizer 8 such as a polysaccharide or polynucleotide and/or an external cross-linker 10 and optionally (iii) an enzyme 12. The matrix protein concentration (gel density), the composition of the matrix protein 4, the concentration of medicinal 6, the shape
25 and size of the gel matrix 2, the amount of polymeric stabilizer 8, the degree of external cross-linking 10 and the amount of enzyme are adjusted to achieve the desired release profile.

Many proteins denature at hydrocarbon-water interfaces. Indeed, to stabilize sensitive proteins often requires inclusion of an "inert" protein such as albumin. Gelatin (collagen) is commonly used to pre-treat surfaces which inactivate or adsorb proteins. Gelatin and albumin are readily available and have
5 very low antigenic potential. Stable dosage forms can be made at moderate temperatures and near neutral pH.

Various types of collagen may be used as the matrix protein 4 in the subject invention, e.g., types A and B, Bloom Nos. 60-300. Advantageously,
10 human collagen is used for human administration.

In addition to gelatin, other non-immunogenic matrix proteins can be used in the subject invention. Serum albumin can be used especially when a strong gel is desired. Human, bovine and rabbit serum albumin may be used.
15 Advantageously, the albumin is native to the animal into which the gel matrix is to be administered. Available amino groups in the form of lysine are high in number in serum albumin. Lysine constitutes approximately 13% of the amino acid composition of serum albumin.

20 In one embodiment both gelatin and serum albumin are used together as the matrix protein. The ratio of these two components can vary, for example 50:50 (w/w), 60:40, 70:30, 80:20 or 90:10 with either protein being present as the primary component.

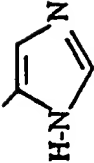

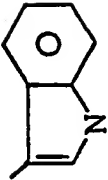
25 Elastin, hemoglobin, myoglobin, serum globins, and proteins of basement membrane can also be used as the matrix protein 4.

The gel matrix 2 can be formed as beads, granules, implants, microspheres (100-200 microns), threads, cylinders, disks, films or cell-sized

microspheres (less than 100 microns) using techniques presented herein and known to those skilled in the art. The gel matrix 2 is typically stabilized internally by cross-linking with the polymeric stabilizer 8, either through ionic bonds, or covalent bonds when the polymeric stabilizer 8 carries amine specific functional groups such as aldehydes and imidates. The gel matrix is optionally
5 stabilized by an external cross-linker 10 such as a multi-functional imidate.

It is useful to stabilize protein gels with covalent cross-links. In solution glutaraldehyde exists in polymeric forms which contain sites for Michael addition
10 reactions with thiols, amines, phenolic hydroxyls, etc.
(see Table 1).

Table 1. Reactivities of functional groups in proteins.

<u>Functional Group</u>	<u>Reagent</u>				
	<u>Acylimidazole</u>	<u>Glutaraldehyde</u>	<u>Diazonium Salts</u>	<u>CHO/NaBH4</u>	<u>Imidates</u>
-NH ₂	+	+	+	+	+
-SH	+	+	+	-	-
	+	+	+	-	-
	-	-	+	-	-
	-	-	+	-	-
-CO ₂ H	-	?	-	-	-
+S-S-	-	-	-	-	-

In the present invention, polyfunctional amine-specific aldehydic and amidination reagents are used to stabilize the matrix in preference to reagents such as glutaraldehyde which are non-specific (see Table 1).

5 Gel Matrix Preparation

Advantageously, the gel matrix 2 components include a matrix protein 4, a polymeric stabilizer 8, a hydrolytic enzyme 12, and an external cross-linker 10. The gel matrix 2 is schematically represented in Figure 1. Gelatin (or other
10 matrix protein) molecules, which are in excess, are not specifically shown. The matrix preparation typically occurs in three steps:

1. The medicinal is mixed with the matrix components (e.g., matrix protein, polymeric stabilizer and enzyme). The medicinal is dissolved or dispersed as an amorphous or crystalline solid. The
15 polymeric stabilizer is optionally included in the formulation prior to gelation. One approach involves formation of a gelatin-polysaccharide bead in a rapidly stirred dispersion in a water-immiscible substance. The anionic polysaccharide can be activated by pre-treatment with sodium m-periodate (Figure 2). The
20 resulting polyfunctional dialdehyde reacts to form primarily internal cross-links.
2. The gel matrix is formed into the desired shape (beads, cylinders, disks, etc.).
3. The solidified matrix is optionally subjected to an external
25 cross-linker to provide added stability and to prolong release.

Multivalent metal cations and/or chemical cross-linkers can be added to cure the outside of the gel matrix. The frequency of chemical cross-links near the outside of the gel is a result of diffusing the bifunctional reagent into a previously formed gel matrix ("curing").

Polymeric Stabilizers

Polysaccharides, such as those listed in the first column of Table 2 are useful as polymeric stabilizers in the gel matrix of the subject invention.

Table 2. Amine specific cross-linking reagents.

<u>Polymeric Stabilizers</u> <u>(Internal Cross-Linkers)</u>	<u>External Cross-Linkers</u>
Dialdehyde-dextran	Imidates
Dialdehyde-dextran sulfate	$\text{MeO} - \overset{\text{NH}_2\text{Cl}}{\underset{\parallel}{\text{C}}} - \text{R} - \overset{\text{NH}_2\text{Cl}}{\underset{\parallel}{\text{C}}} - \text{OMC}$
Dialdehyde-Chondroitin Sulfate	
Dialdehyde-Hyaluronic Acid	$\text{R} = (\text{CH}_2)_n \text{ or } (\text{CH}_2\text{CH}_2\text{O})_n$

Chondroitin sulfate and hyaluronic acid are immunologically inert. Strong gels can be formed using polyanions such as polysaccharides in combination with multivalent metal ions and polymeric cations (Joung, J.J., et al, Appl. Biochem. Biotechnol., 14, 259 (1987)). In addition to chondroitin sulfate and hyaluronic acid, dextran (including oxidized dextran, i.e., dextran-CHO) and dextran sulfate have been used successfully. Clinical grade dextran has the advantages of low cost and ease of handling. Polynucleotides are also useful as polymeric stabilizers.

10 The degree of internal cross-linking can be varied. The rate of release is inversely related to the degree of cross-linking.

External Cross-Linkers

15 Once the gel matrix is formed into the desired shape, treatment with an external cross-linker (i.e., curing the gel matrix) is desirable where added physical stability and prolonged release are needed. For example, beads prepared either in water or in organic medium, can be subjected to curing by soaking the beads in a solution of diimide. The rate of release is inversely related to the degree of external cross-linking.

Advantageously, amine-specific cross-linkers are used in the subject invention. Diimides form stable amidine adducts with amino groups of proteins and are especially useful as external cross-linkers. Advantageous compounds useful as external cross-linkers are presented in the second column of Table 2.

For delivery of non-proteinaceous drugs, other less specific cross-linkers are also useful. Examples include: multifunctional alkylating agents, multifunctional acylating agents, and multifunctional carbonates such as



in which X is a leaving group such as a halide, a phenol or hydroxy succinimide. If a hydrolytic enzyme is to be included, it must be pre-treated with an analogous
 10 mono-functional reagent to avoid covalent immobilization within the matrix. An example of the latter reagent is acetyl imidazole.

In one embodiment of the invention, only an external cross-linker is used, i.e., polymeric stabilizer is omitted.

15

* * *

Sub-batches of beads (or other shapes) can be prepared by cross-linking (either using internal or external cross-linkers) for varying time periods. For
 20 example, beads can be made where 10, 20, 30, 40, 50, 60, 70, 80 or 90 percent of available amino groups are subject to cross-linking. These sub-batches can be used to constitute blends of beads. To illustrate, when these blends of beads contain proportionately more of the heavily cross-linked sub-batch, the release is relatively slow. Blends of beads weighted toward lightly cross-linked sub-
 25 batches release drug relatively quickly.

Enzymes

Programmable erosion of the gel matrix (and timed release of the medicinal) is made possible in the present invention by including one or more hydrolytic enzymes in the gel matrix. As an example, when beads are chosen as the gel form, the hydrolytic enzyme can be included directly into the proteinaceous bead containing the drug, in companion beads, or added to the formulation as free enzyme.

Conditions for the preparation and storage of gel matrix must be selected to avoid enzymatic degradation prior to use. It is well known that enzyme activity is strongly dependent on pH, temperature, ionic strength, and the presence of modifiers. Conditions such as low temperature and pH can be selected to suppress enzyme activity. Lyophilization can also be used to suppress enzyme activity.

Enzymes may react with the cross-linker in a manner similar to the medicinal protein, and should therefore be protected. It is well known that amino groups of enzymes can be modified without loss of activity. Therefore, pretreatment of the enzyme according to methods described below with respect to the medicinal proteins (see Figure 6) may be carried out on enzymes prior to direct incorporation.

In a second embodiment, the hydrolytic enzymes are included in companion gel matrix beads as shown in Figure 3. In this case the population

of the drug-containing gel matrix beads 2 is homogeneous. The enzyme 12 containing beads 2 are blended to achieve the desired release profile. Companion beads 2 with high enzyme content and low degree of cross-linking would produce fast release. In contrast, companion beads 2 with low enzyme content
5 and high degree of cross-linking would result in slow release.

In a third embodiment, hydrolytic enzymes 12 are added directly to the formulation in soluble or in crystalline form (see Figure 4). Gel matrix beads 2 and enzyme 12 are mixed just prior to administration or stored together in the
10 dosage form under conditions which do not support enzyme activity such as low ionic strength, low pH, or absence of activators. In a variation of this embodiment enzymes are also present in the gel matrix.

In a fourth embodiment of the invention, an implant or capsule 14 is
15 constructed as shown in Figure 5. A blend of gel matrix beads 2 with varying amounts of cross-linking is included in the advantageously low density gel capsule or implant 14. Prolonged release is achieved by weighting the blend in favor of highly cross-linked gel matrix beads 2. Also, collagenase (enzyme containing) beads 2 can be included to modulate release rate. The implant or
20 capsule 14 may have a semipermeable membrane (for use as an implant) or enteric coating (for oral use).

An advantageous enzyme is collagenase, which digests the gelatin matrix component. Alternatively, an enzyme specific for the polymeric stabilizer is
25 used. Hyaluronidase, dextranase, or nuclease represent this latter type of enzyme.

Digestion of the gel matrix has two results—reduction of the viscosity of the medium through which the protein medicinal must travel and perforation of the gel matrix surface. The hydrolytic enzymes must have narrow specificities which exclude the medicinal protein.

5

Purified collagenase selectively cleaves after X in the sequence PRO-X-GLY-PRO where X is any neutral amino acid. PRO can designate either proline or hydroxyproline. GLY represents glycine. Although very frequent in collagen, the sequence, PRO-X-GLY-PRO is generally rare in proteins.

10

Hyaluronidase cleaves glycosaminoglycans but not polypeptides. It catalyzes the hydrolysis of glycosidic bonds of beta-N-acetyl-hexosamine (1,4-linked).

15

Gels containing relatively high amounts of hydrolytic enzymes will permit faster release of the medicinal. Desired delivery profiles can be produced by blending batches of beads with varying amounts of enzyme. In one embodiment more than one type of enzyme is included in the gel matrix, e.g., dextranase and collagenase.

20

Medicinal Proteins

As used herein the term medicinals includes proteins as well as small molecule agents. The term "protein" includes naturally occurring proteins, recombinant proteins, protein derivatives and polypeptides.

25

Medicinal proteins useful in the subject invention include colony stimulating factors (CSF) including G-CSF, GM-CSF, and M-CSF; erythropoietin; interleukins, IL-2, IL-4, IL-6, etc; interferons; growth factors (GF) including epidermal-GF, nerve-GF; tumor necrosis factor (TNF); hormones/bioactive
5 peptides; ACTH; angiotensin, atrial natriuretic peptides, bradykinin, dynorphins/endorphins/ β -lipotropin fragments, enkephalin; gastrointestinal peptides including gastrin and glucagon; growth hormone and growth hormone releasing factors; luteinizing hormone and releasing hormone; melanocyte stimulating hormone; neurotensin; opiate peptides; oxytocin, vasopressin and
10 vasotocin; somatostatin; substance P; clotting factors such as Factor VIII; thrombolytic factors such as TPA and streptokinase; enzymes used for "replacement therapy," e.g., glucocerebrosidase, hexosaminidase A; and antigens used in preventative and therapeutic vaccines such as tetanus toxoid and diphtheria toxoid.

15

Protection of the Medicinal Proteins

In order to avoid chemical cross-linking of the medicinal protein within the matrix, prior to exposing the therapeutic protein to the cross-linking reagent,
20 the bioactive protein is modified to deactivate functional groups which would normally react with the cross-linking agent, i.e., the protein is "protected.". In the case of certain proteins, there is no need to modify the protein for it to be protected and useful in the subject invention, e.g., a protein where amino groups are not available. Since the cross-linkers of choice attack primary amino groups,
25 these groups are protected in the medicinal protein. For example, the protein is

first rendered unreactive to amine-directed reagents by treatment with formaldehyde and a reducing agent, or by other suitable reagents, such as methyl acetimidate. The charge, structure and biological activity of the reductively alkylated protein is not significantly changed but the procedure prevents further
5 reaction with aldehydes or other reactions such as acylation or amidination.

Reductive methylation and amidination are examples of appropriate reactions for amino group protection. These treatments are known to have minimal effect on biological activity probably because of the minor structural
10 change and similar net charge of the reactant and product at neutral pH (Means, G.E., Methods Enzymol., 47, 469 (1977)). Both techniques are convenient and give consistent results. Antigenicity of the reductively methylated proteins is not enhanced over native structures.

15 Other methods of amino group protection include (i) acylation, (ii) glycosylation and (iii) carbamylation.

Examples of acylation reactions are:

- 20
1. acetylation
 2. maleylation
 3. citraconylation
 4. trifluoroacetylation
 5. acetoacetylation

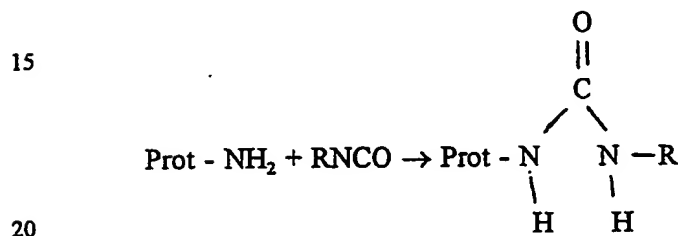
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 6. ethoxyformylation

Examples of glycosyl groups include:

1. glucosyl
2. lactosyl
- 5 3. galactosyl
4. mannosyl
5. maltosyl
6. fructosyl
7. arabinosyl
- 10 8. fucosyl
9. combinations of the above

Carbamylation reactions are also appropriate for amino group protection:



R can be hydrogen, in which case the reactant is cyanate and the product is a substituted urea.

25 There are numerous other amino group modifications known to those skilled in the art such as guanidination and sulfonylation. Non-specific alkylating reagents are to be avoided. However, where stable derivative favor amine adducts, this class of reagents may be useful.

Examples of protected medicinal proteins are dimethylated and amidinated derivatives of the proteins listed above under "Medicinal Proteins", e.g., dimethylated G-CSF or dimethylated IL-2. It should be noted that the lysines in some proteins are not available for reaction due to location. There is no need for
5 these locations to be protected since the same locations are inaccessible to cross-linking.

Non-Protein Delivery

Although especially well suited for parenteral administration of proteins,
10 the present delivery system is also applicable to formulations with non-protein medicinals, including but not limited to alkaloids, steroids, terpenoids, amino acid derivatives, nucleoside/nucleotide derivatives, polynucleotides, carbohydrates, polysaccharides, lipids, lipopolysaccharides, purines, pyrimidines and derivatives of same.

15

Advantageous small molecule drugs include: analgesics, anesthetics, antialcohol preparations, anti-infectives, anticoagulants, anticancer drugs, antidepressants, antidiabetic agents, antihypertensive drugs, antiinflammatory agents, antinauseants, anorexics, antiulcer drugs, cardiovascular drugs,
20 contraceptives, decongestants, diuretics, hormones/antihormones, immunosuppressives, narcotic detoxification agents, uncosuric agents, agrichemicals such as pheromones, wood protection chemicals and wound healing promoters.

Particularly advantageous compounds for use in the subject invention are those in crystalline form.

Films for transdermal delivery or for topical application as bandages can
5 also be formed. In this case the film may be used to deliver non-proteinaceous drugs such as anti-infectives and wound healing promoters.

* * *

10 Typical delivery systems are shown in Table 3:

TABLE 3

<u>Matrix Protein¹</u>	<u>Polymeric Stabilizer</u>	<u>Hydrolytic Enzyme²</u>	<u>External Cross-Linker</u>
G = 100% A = 0	Dextran-CHO	Collagenase	DMS ³
G = 100% A = 0	Chondroitin sulfate	Collagenase	DMS
G = 100% A = 0	Chondroitin sulfate	Hyaluronidase	DMS
G = 50% A = 50%	Dextran-CHO	Collagenase	DMS
G = 50% A = 50%	Dextran-CHO	Hyaluronidase	DMS
G = 25% A = 75%	Dextran-CHO	Dextranase	---
G = 25% A = 75%	Chondroitin sulfate	Hyaluronidase	DMS
G = 50% A = 50%	Polynucleotide	Nuclease	DMS
G = 50% A = 50%	----	Collagenase	DMS
G = 50% A = 50%	Dextran-CHO	Collagenase	---

¹ G = gelatin (collagen) and A = albumin

² Delivered either by the same matrix (internal) or by a different matrix (external)

³ Dimethyl Suberimide

Timed Release of Medicinals and Modes of Administration

An idealized release profile is shown in Figure 7. Here the concentration of medicinal in the vicinity of the bead reflects the rate of internal degradation of the three different classes of gel matrices. The profile shown depicts the system with identical medicinal concentration in all classes. For example, to have higher levels of medicinal released at a later period, more medicinal would be incorporated in the Class III beads as shown in this example.

Release profiles can be obtained from zero order release to those involving late-stage bursts.

It is also possible to administer more than one medicinal in the same treatment regimen. The drugs could be released simultaneously or sequentially.

Stokes law is applicable, i.e.,

$$D \propto 1/rv$$

The diffusion coefficient (D) is inversely related to the radius of the protein (r) and the viscosity of the medium (v), which is dependent on the density and degree of cross-linking of the gel matrix. Computer modelling of this system with four to seven adjustable parameters can be used to generate a set of hypothetical release profiles for a given therapeutic protein.

Medicinal matrix of the invention is administered to a human or other mammal as beads, disks, threads and implants of various other shapes using techniques known to those skilled in the art. Beads would be normally administered via needle subcutaneously, intramuscularly, intraperitoneally, or
5 intravenously for cell-sized microbeads. Tablets and capsules are used for oral delivery.

Medicinal matrix can be administered concomitant with surgical procedures. Examples include 1) an antibiotic matrix following abdominal
10 surgery, 2) matrix containing cytotoxic chemotherapeutic drug following tumor removal, and 3) matrices containing adjuvants/antigens following tumor removal. Also, implants can be surgically placed under the skin or elsewhere.

Matrices designed for enzyme-catalyzed erosion are excellent, convenient
15 substrates for enzyme assays when the medicinal protein is replaced by a polymeric chromophore (fluorophore). Gelatin/casein matrices cross-linked with oxidized dextran are convenient solid substrates for a wide range of proteases, including papain, trypsin, chymotrypsin, thermolysin, pepsin, subtilisin, etc. Blue dextran is an ideal polymeric chromophore as the background readings are zero
20 even after 24 hours.

Cross-linked matrices of polysaccharides or nucleic acids can be used analogously as substrates for polysaccharide hydrolases and nucleases, respectively.

The following Examples are illustrative, but not limiting of the compositions and methods of the present invention. Other suitable modifications and adaptations of a variety of conditions and parameters normally encountered in clinical therapy which are obvious to those skilled in the art are within the spirit and scope of this invention.

EXAMPLES

Example 1

10

Production of Protected Proteins for Use in the Delivery Systems

Reductive methylation of the protein is carried out with formaldehyde and sodium borohydride at 0-4°C in 0.2 M borate buffer, pH 9. In cases where lower pH is desirable, sodium cyanoborohydride is used in place of sodium borohydride at pH values below pH 9. The protein concentration is 1-10 mg/ml. The borohydride is added in advance of the formaldehyde with moderate stirring. For each milliliter of protein solution, 0.5 mg borohydride is added followed by 2.5 microliters of formaldehyde solution (37%) in five increments at five minute intervals. The procedure is repeated if necessary. The modified protein is purified by dialysis or gel filtration.

Example 2

Preparation of Activated Polysaccharide

5 Generation of dialdehydes from diols by using periodate oxidation is accomplished as follows. The reaction is carried out in the pH range of 4.5-6.5. A polysaccharide, such as chondroitin sulfate, hyaluronic acid, dextran, dextran sulfate, or the like, is dissolved in distilled water (0.1-10mg/ml). An equal volume of sodium metaperiodate solution (1-50 mM) is added and the mixture is
10 maintained at room temperature in the dark for about an hour. Higher temperature and longer reaction time result in more extensive oxidation. The activated polymer is purified by dialysis, gel filtration or ultrafiltration. Clinical grade dextran (60,000 - 90,000 MW) oxidized to the extent of 20% can be stored (0-5°C) and used as a 10% stock solution for at least a week.

15

Example 3

Preparation of Molded Gels Containing Bioactive Proteins

20 Many different shapes are possible because of the reaction dynamics and their control. A cylindrical implant is conveniently made by using a plastic syringe. The syringe is filled with reaction mixture which is then allowed to set. The constricted end is cut off and the plunger is depressed to expel the gel cylinder.

25

A reaction mixture is as follows: Solution I-Type A gelatin (0.5-5g) is dissolved in hot Hepes buffer (50 ml, 10mM pH 7-8.5) and then cooled to 37°C. Solution II-to an activated polysaccharide solution (0.05-1% in 10mM Hepes, pH 7-8.5) is added the protected protein (and enzyme if included in the formulation).
5 Protected protein concentration is in the range of 10-2000 micrograms/ml and the protected hydrolytic enzyme concentration is in the range of 1-50 micrograms/ml. The reaction mixture is composed of equal volumes of Solution I (gelatin) and Solution II (polymeric stabilizer) described above. The two solutions are mixed with a dispo pipette and loaded into the syringe. The gelation occurs at room
10 temperature in 2-5 hr. The cylinder is forced out in one piece or incrementally forced out and cut into disks of desired thickness. These cylinders or disks are optionally further stabilized by soaking in a solution containing an amine specific cross-linking reagent such as dimethylsuberimide (10 mM Hepes, 1-100 mM imide, 1-5 hr). The surface curing reaction is terminated by pouring off the
15 imide solution and quenching with 0.1 M aminoethanol-HCl, pH 8.5 for 1 hour.

Example 3A

Films

20

The procedure of Example 3 is repeated using other geometric configurations including films containing antibiotics and wound healing promoters. The reaction mixture is poured onto a flat glass plate with borders to provide boundaries of the desired dimensions. The glass plate is cooled to -20°C
25 and allowed to stand for 4-8 hours.

Example 4

Formation of Microbeads in a Non-miscible Solvent

5 For formation of microbeads, the Hepes buffer contains 0.1% sodium dodecyl sulfate, and the reaction mixture (as above) is injected into a rapidly stirring hydrocarbon phase (corn oil:petroleum ether-4:1, 100 ml, 0-4°C). After an hour, the beads are harvested, washed with petroleum ether, and surface cured as described above. The resulting beads are about 100 microns in diameter.

10

Example 4A

Beads Containing Dispersed Solid Medicinal

15 The procedure of Example 4 is repeated but with 10-40% by weight of a crystalline or amorphous solid medicinal suspended in the reaction mixture.

Example 5

20 Microsphere Formation in Water

 The basic gelatin stock solution (5ml) is mixed with activated polysaccharide with rapid stirring. The concentration of activated polysaccharide is in the range of 0.1-1%. The protected protein is included in the original
25 solution of the activated polysaccharide within the concentration range of 1-5000

micrograms/ml. These microspheres are collected by centrifugation and surface cured as described above.

Example 6

5

Controlled Release of Azoalbumin from Three Preparations

Controlled release was demonstrated using three preparations made as disks (2x6mm in diameter) according to Example 3. Class I contained mildly
10 oxidized dextran and was not treated with external cross-linkers; Class II contained intermediate levels of dextran-dialdehyde and was externally cross-linked for thirty minutes with an intermediate concentration of dimethylsuberimidate (DMS). Class III contained the maximum activated polysaccharide and was soaked in 0.1 M DMS for five hours. Each preparation
15 contained Type A gelatin/Bloom # 60/1.5% final concentration and 2% protected azoalbumin. All were treated with 200 micrograms of collagenase in one ml of Tris buffer (10 mM, pH 7.4, 1 mM CaCl_2). The results are shown in Table 4.

Table 4

	Class I	Class II	Class III
5			
	<5 min	2 wks	negligible release over 1 month
10			

Example 7Preparation of a Cross-Linked Matrix Comprising Gelatin, Casein and
a Polymeric Chromophore. Said Matrix Being Useful as a Protease Substrate

15

Although a wide variety of compositions will produce stable gels, the following preparation has a number of advantages: 1) it is susceptible to proteolysis by many enzymes, including collagenase, 2) the assay backgrounds are zero even for 24 hours as long as the assay buffer contains 0.1% azide, and

20 3) the matrix is physically and thermally stable to 100°C.

The following reagents are used:

1. Casein (Sigma product # c8645) 11% in water
2. Gelatin (Sigma product # g6144) 11% in water-37°C
- 5 3. Dextran (Sigma product # d4751) 10% in water, oxidized to the extent of 20% according to Example 2. As an alternative, cross-linker glutaraldehyde may be used (.125%)
4. HEPES, 0.1M in water
- 10 5. Blue dextran/solid (Sigma product # d5751)

Procedure:

- 15 Mix 2 ml each of gelatin and casein solution with 1 ml HEPES warming to about 40°C. Add 50 mg blue dextran and allow this to dissolve. With rapid mixing add 0.5 ml of glutaraldehyde solution. Dispense into multi-well plates or screw cap vials with a repeat pipettor.

20 Example 8

Assay of Proteases Using the Above Matrix

- To exemplify the hydrolase assay, 200 ul of the matrix described in
- 25 Example 7 is placed in a 5-ml screw-cap vial. The assay buffer is HEPES, 10 mM, containing 10 mM calcium chloride and 0.1% sodium azide. The enzyme in this example is pronase (Sigma pr #5147) at the levels indicated below; assay volume equals 1ml. The vials are incubated at 37°C and the absorbance (595 nm) determined as a function of time. The results follow for three enzyme
- 30 levels:

	Time (hr)	Enzyme/vial	1ug Absorbance	10ug 595 nm	100ug
5	1		0.031	0.162	0.399
	2		0.074	0.271	0.687
	3		0.134	0.427	0.884
	4		0.176	0.531	1.040
	5		0.232	0.641	complete

10

Similar results can be generated using multi-well plates and with an automatic reader. In the latter case, substrate is used at the level of 50 ul/well.

EXAMPLE 9

15

In another embodiment, the assay can be formatted for a fixed time period, varying levels of substrate, and visualization as opposed to instrumental measurements.

20

Individual 8-well strips or 96-well plates can be prepared with either colored or fluorescent substrates. The visual determination of activity is surprisingly accurate especially when a UV or white light transilluminator is used. One novel format employs varying substrate thickness and a single reading at a preset time (Fig. 8). At the end of the time period the strip or plate is washed and viewed with the aid of a transilluminator. The first strip/plate position devoid of color or fluorescence correlates with the amount of enzyme present. To illustrate, if positions 6, 7 and 8 (Fig. 8) were blank, a relatively-small amount of enzyme is present. If positions 4-8 were blank, a relatively-high

25

amount of enzyme was present. Calibration with an enzyme standard is required for precise quantitation.

* * *

5

It will be readily apparent to those skilled in the art that numerous modifications and additions may be made to both the present invention, the disclosed device, and the related system without departing from the invention disclosed.

WHAT IS CLAIMED IS:

1. A gel matrix medicinal delivery system comprising
 - (i) at least one matrix protein selected from the group consisting of gelatin and albumin,
 - (ii) a polymeric stabilizer and/or an external cross-linker, and optionally
 - (iii) an enzyme capable of degrading said protein or said polymeric stabilizer,wherein said system is stabilized by said matrix protein being cross-linked with said polymeric stabilizer and/or said external cross-linker.
2. A system as in Claim 1 further comprising a medicinal protein.
3. A system as in Claim 1 wherein said polymeric stabilizer is selected from the group consisting of dextran, hyaluronic acid, chondroitin sulfate, dextran sulfate or polynucleotides.
4. A system as in Claim 1 wherein said external cross-linker is an amine specific reagent.
5. A system as in Claim 4 wherein said external cross-linker is a diimide selected from the group consisting of dimethyl suberimide, pimelimide, dimethyladipimide and suberic acid bis(N-hydroxy succinimide).

6. A system as in Claim 2 wherein said enzyme and said medicinal protein are protected.
7. A system as in Claim 1 wherein said matrix protein is gelatin, said polymeric stabilizer is dextran-CHO and said enzyme is collagenase.
8. A system as in Claim 1 wherein said matrix protein is gelatin, said polymeric stabilizer is chondroitin sulfate, and said enzyme is collagenase.
9. A system as in Claim 1 wherein said matrix protein is gelatin and albumin, said polymeric stabilizer is dextran-CHO, and said enzyme is collagenase.
10. A system as in Claim 1 wherein said matrix protein is gelatin and albumin, said polymeric stabilizer is chondroitin sulfate and said enzyme is hyaluronidase.
11. A system as in Claim 1 wherein said polymeric stabilizer is a polynucleotide and said enzyme is a nuclease.
12. A system as in Claim 1 wherein said system is in the form of a bead.
13. A system as in Claim 1 wherein component (i) comprises 0-80% by weight albumin and 0-80% by weight gelatin.

14. A system as in Claim 1 wherein less than 20% of the available gelatin and/or albumin amino groups are cross-linked.

15. A sustained release delivery system comprising:

(a) a first gel matrix comprising

(i) a matrix protein selected from the group consisting of gelatin and albumin, and

(ii) a polymeric stabilizer and/or an external cross-linker, and

(iii) a medicinal protein

wherein said first gel matrix is stabilized by said matrix protein being cross-linked to said polymeric stabilizer and/or said external cross-linker, and

(b) a second gel matrix comprising

(i) a matrix protein selected from the group consisting of gelatin and albumin, and

(ii) a polymeric stabilizer and/or an external cross-linker, and

(iii) an enzyme capable of degrading said matrix protein or said polymeric stabilizer,

wherein said second gel matrix is stabilized by said matrix protein being cross-linked to said polymeric stabilizer and/or said external cross-linker.

16. A medicinal delivery system comprising

(i) at least one matrix protein selected from the group consisting of gelatin and albumin, and

- (ii) a polymeric stabilizer and/or an external cross-linker, wherein said system is stabilized by said matrix protein being cross-linked with said polymeric stabilizer and/or said external cross-linker, and
- (iii) an enzyme capable of degrading said matrix protein or said polymeric stabilizer embedded on the surface of said system.

17. A delivery system as in Claim 15 wherein said enzyme and medicinal protein are protected.

18. A blend of gel matrices for sustained release comprising at least two gel matrices according to Claim 1 wherein at least two of said gel matrices have different levels of internal cross-linking.

19. A blend of gel matrices for sustained release comprising at least two gel matrices according to Claim 1 wherein at least two of said gel matrices have different levels of external cross-linking.

20. A blend of gel matrices for sustained release comprising at least two gel matrices according to Claim 1 wherein at least two of said gel matrices have different levels of enzyme.

21. A blend of gel matrices for sustained release comprising at least two gel matrices according to Claim 1 wherein at least two of said gel matrices have different levels of gel density.

22. A method for obtaining sustained release of a medicinal protein comprising administering the system of Claim 1 to a mammal.

23. A method for obtaining sustained release of a medicinal protein comprising administering the system of Claim 18 to a mammal.

24. A method as in Claim 22 wherein said administration is oral.

25. A protected medicinal protein selected from the group consisting of dimethylated G-CSF and dimethylated IL-2.

26. A method of protecting a protein from reaction with amino groups on said protein comprising:

reacting said protein with borohydride and formaldehyde or acetone.

27. A method of synthesizing a drug delivery system comprising the steps of:

- a) mixing matrix protein, protected medicinal protein, hydrolytic enzyme, and polymeric stabilizer to form a gel matrix,
- b) shaping said gel matrix, and optionally
- c) curing said gel matrix with an external crosslinker.

28. A method of assaying hydrolytic enzymes in a sample comprising the steps of

contacting a sample which may contain a hydrolytic enzyme with gel matrix containing a marker, and
determining the amount of marker which has been released from said gel matrix.

29. A method as in claim 28 wherein said hydrolase is a protease.

30. A method as in claim 28 wherein said marker is a polymeric chromophore or a fluorophore.

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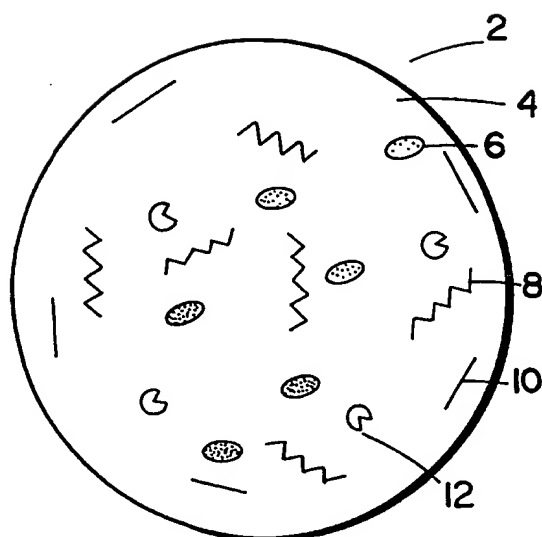


FIG. 1

SUBSTITUTE SHEET (RULE 26)

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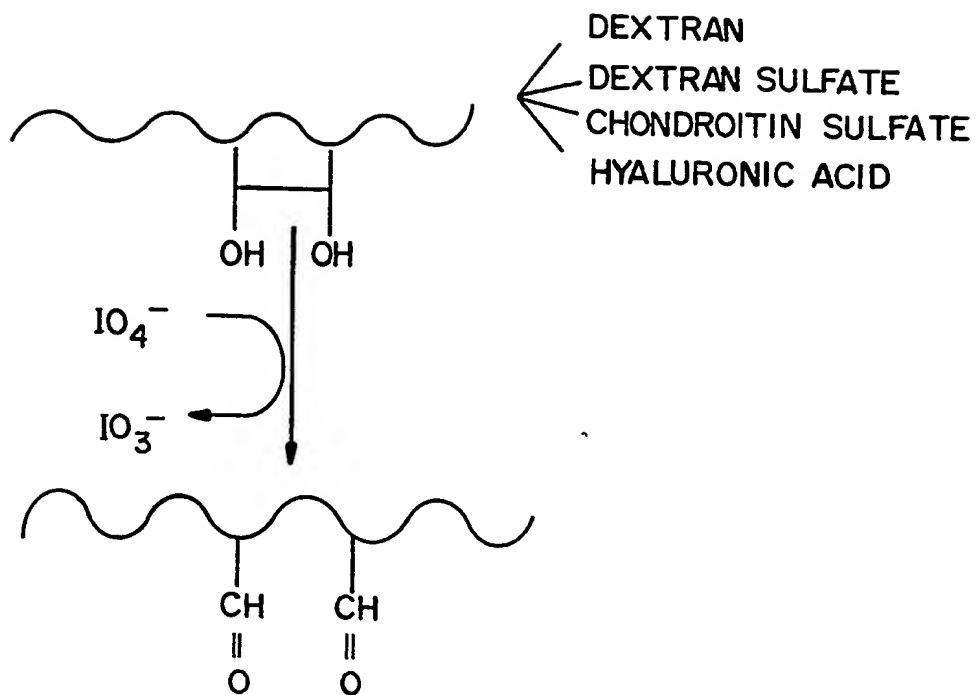


FIG. 2

SUBSTITUTE SHEET (RULE 26)

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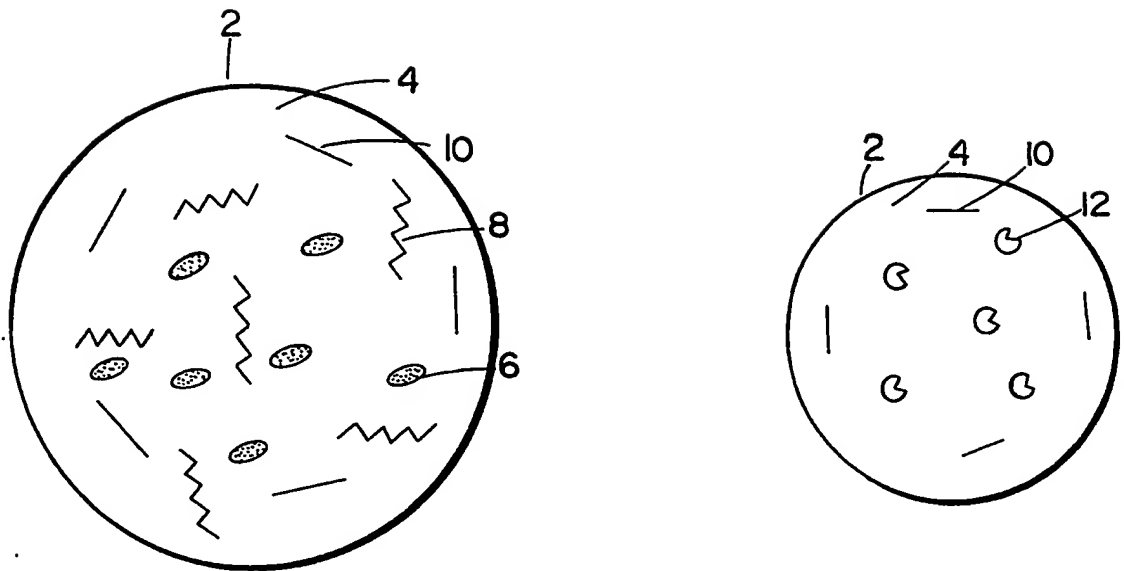


FIG. 3

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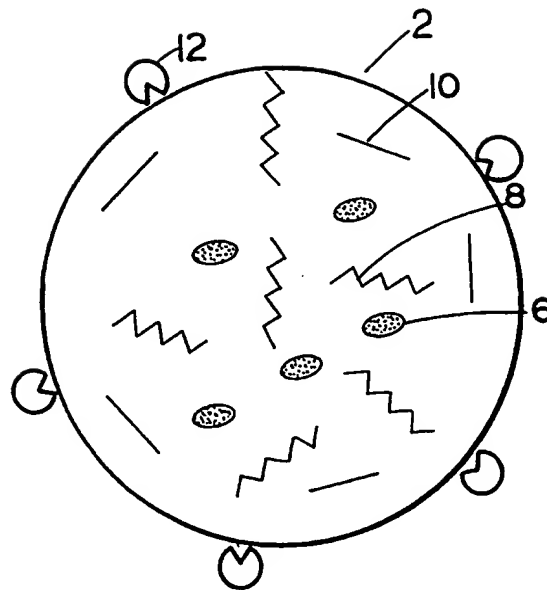


FIG. 4

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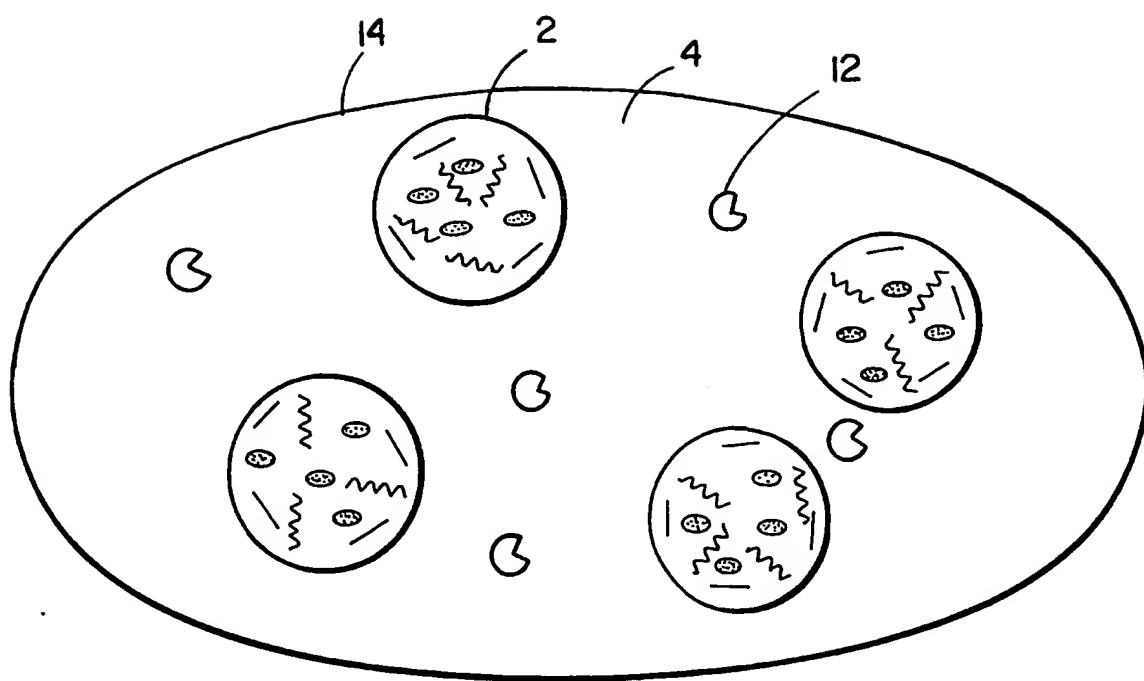


FIG. 5

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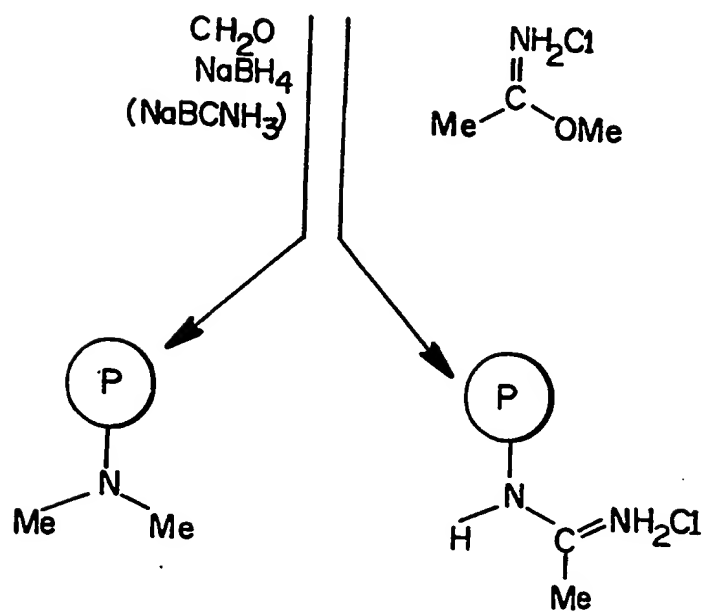
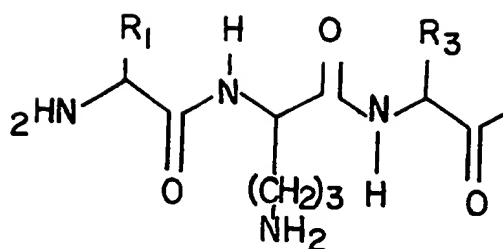


FIG. 6

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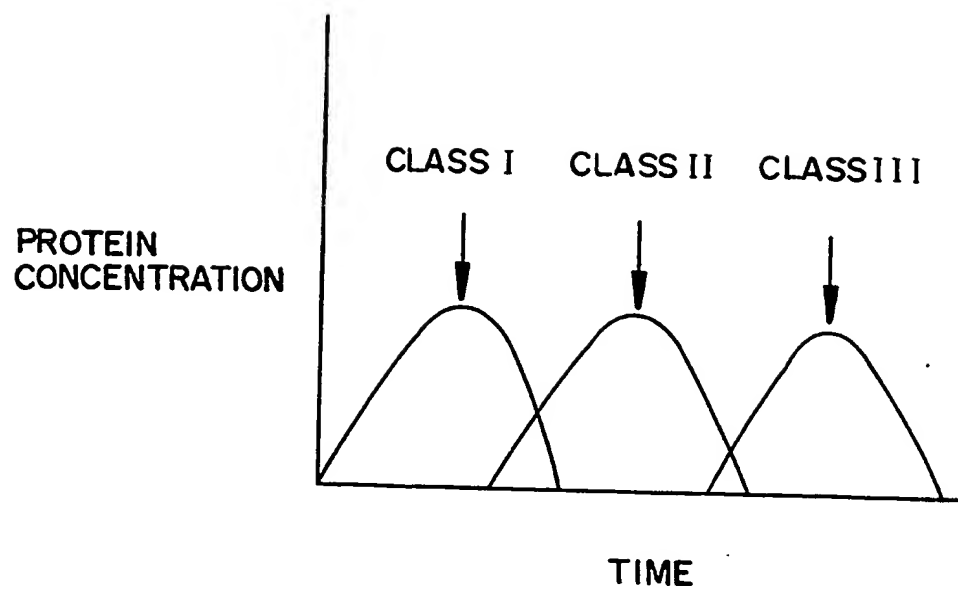


FIG. 7

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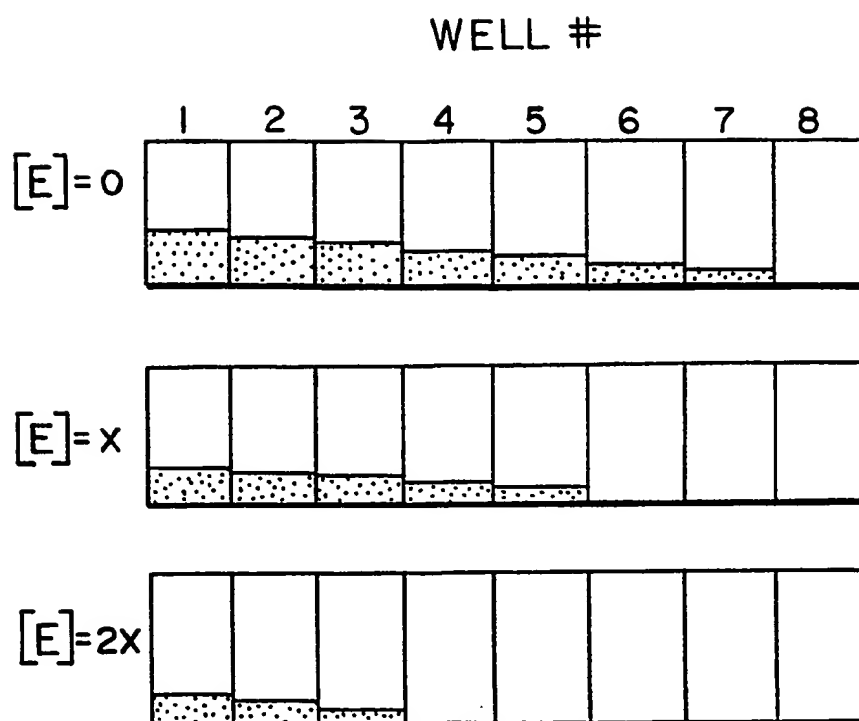


FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07186

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61L 15/16; A61F 13/02

US CL :424/444, 448

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/444, 448

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US, A, 5,041,292 (FEIJEN) 20 August 1991, see the entire document.	1-4, 12-14, 22-24, 27 ----- 5, 7-10, 15-21
Y	US, A, 3,925,344 (MAZUR) 09 December 1975, see the entire document.	5

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

18 JULY 1995

Date of mailing of the international search report

08 SEP 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/07186

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-24 and 27

Remark on Protest

☐
☐

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07186

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

I. Claims 1-24 and 27, drawn to gel matrices

II. Claims 25-26, drawn to alkylated proteins

III. Claims 28-30, drawn to assay methods

The inventions listed as Groups I-III do not meet the requirements for unity of invention for the following reasons:

Inventions II and I are related as intermediate-final product and the intermediate has utility to make products other than the final product of Group II such as radioactive labels for physiological/metabolic studies.

Inventions II and III are independent in that there exists no common technical feature between them.

Inventions I and III are related as product and process of use and the product has utility to make products other than the final product of Group III such as devices for programmed drug delivery.

Inventions I, II and III do not have a common technical feature and therefore lack utility under PCT Rule 13.1.